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DOI: <https://doi.org/10.1016/j.semcancer.2013.03.001>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-90079>

Journal Article

Accepted Version

Originally published at:

Romao, Susana; Gannage, Monique; Münz, Christian (2013). Checking the garbage bin for problems in the house, or how autophagy assists in antigen presentation to the immune system. *Seminars in Cancer Biology*, 23(5):391-396.

DOI: <https://doi.org/10.1016/j.semcancer.2013.03.001>

**Checking the garbage bin for problems in the house,
or how autophagy assists in antigen presentation to the immune system**

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Keywords: MHC class I, MHC class II, phagocytosis, exocytosis, T cell selection, CD4⁺ T cells, CD8⁺ T cells

Abstract

Macroautophagy was originally discovered as a nutrient salvage pathway during starvation. By now it has not only become clear that degradation of cytoplasmic constituents via transport by autophagosomes to lysosomes can be used for innate and adaptive immunity, but that the core machinery assists antigen presentation to the immune system by a variety of vesicular transport pathways. All of these rely on the presentation of small protein waste fragments, which are generated by a variety of catabolic pathways, including macroautophagy, on major histocompatibility complex (MHC) molecules. In this review, we will point out how classical macroautophagy, as well as phagocytosis and exocytosis, which both benefit from the core autophagic machinery, assist in antigen presentation on MHC class I and II molecules to CD8⁺ and CD4⁺ T cells, respectively. Finally to high-light that macroautophagy is always intimately interconnected with cell death in addition to the various supported vesicular transport function, its role in lymphocyte, especially T cell, development and function will be discussed. From this body of work a picture is emerging that the core machinery of macroautophagy can be used for a variety of vesicular transport pathways and to modulate cell survival, besides its classical role in delivering intracellular material for lysosomal degradation.

1. Introduction

The adaptive immune system hinges on the orchestration by CD4⁺ helper T cells and cytotoxic CD8⁺ T cells as main effectors of cell-mediated immunity. These lymphocyte populations do not recognize antigen directly, but their T cell receptors detect antigen fragments on major histocompatibility (MHC) class I molecules for CD8⁺ T cells and MHC class II for CD4⁺ T cells. These fragments, peptides of eight or nine amino acids in length for MHC class I and longer, often N- and C-terminal extensions of a nonameric core sequence for MHC class II, are generated and loaded onto MHC class I and II molecules in different cellular compartments [1, 2]. MHC class I ligands are in their majority generated by the proteasome, a multicatalytic protease in cytosol and nucleus. In contrast, peptides for MHC class II loading are primarily produced in lysosomes. Loading of MHC class I and II molecules occurs primarily in the endoplasmic reticulum (ER) and late endosomes, respectively. Both molecules are co-translationally inserted into the ER. MHC class I ligands of proteasome origin get transported into the ER by the transporter associated with antigen processing (TAP) and loaded onto MHC class I molecules in the MHC class I loading complex, containing aminopeptidases, chaperones and protein disulfide isomerases. Once a high affinity peptide is loaded into the peptide binding groove of MHC class I, the complex is transported to the cell surface for CD8⁺ T cell recognition. Peptide binding to MHC class II molecules in the ER is prevented by the chaperone invariant chain (Ii), which blocks the peptide binding groove and contains in its cytosolic domain motifs that will facilitate the transport to late endosomes, either via the cell membrane or directly. These late endosomes that are equipped with the MHC class II loading machinery are called MHC class II containing compartments (MIICs). Lysosomal proteolysis within these compartments degrades Ii until only the peptide binding groove contains the last Ii remnant, called MHC class II associated invariant chain peptide (CLIP). CLIP is then exchanged for a high affinity peptide with the help of the chaperone HLA-DM or H2-M. In some cell types like B cells the negative regulator HLA-DO or

H2-O modifies this event. MHC class II complexes, stabilized by high affinity peptides, then migrate to the cell surface in order to engage CD4⁺ T cells.

Access to MHCs or proteasomes, maybe even specific immunoproteasomes, decides how efficient antigens can be loaded onto MHC molecules for T cell stimulation. Due to their co-localization, cytosolic and nuclear antigens are preferentially processed for MHC class I presentation, and endocytosed antigens efficiently reach MHCs. Thus, in the classical paradigm of antigen processing, intracellular antigens are loaded onto MHC class I, while extracellular antigens are preferentially processed for MHC class II presentation. However, there are exceptions to this rule. In specialized antigen presenting cells, like dendritic cells (DCs), extracellular antigens can gain access to MHC class I presentation. This pathway is called cross-presentation [3]. During cross-presentation, antigen is thought to escape from early endosomes or even after endosome fusion with the ER into the cytosol for proteasomal degradation. Vice versa, intracellular antigen fragments can be found on MHC class II molecules and autophagy contributes to this processing as will be discussed in the next chapter.

2. Delivery of intracellular antigens for MHC class II presentation via autophagy

Evidence of intracellular antigen processing onto MHC class II molecules originally came from peptide elution studies, which were aimed at characterizing the ligand repertoire that is presented to CD4⁺ T cells. These studies revealed that up to one third of eluted MHC class II ligands originate from cytosolic and nuclear source proteins [4-6]. MHC class II presentation of these cytosolic and nuclear antigens can be enhanced by starvation, which up-regulates macroautophagy [4]. Among these peptides, the essential autophagy proteins LC3 and GABARAP were found [4, 7]. These are mammalian orthologs of the ubiquitin-like autophagy related gene (Atg) 8 protein that get coupled to the membranes of autophagosomes, the characteristic double-membrane vesicles of macroautophagy, during its formation around its substrates [8]. This conjugation is mediated by the E3-like ligase composed of Atg5, Atg16L and

the ubiquitin like protein Atg12. Interestingly, Atg8 is the only macroautophagy protein that stays with the completed autophagosome, specifically on the inner autophagosome membrane, while all Atgs are recycled from the outer membrane upon autophagosome completion (Figure 1). Therefore, the Atg8 homologues LC3 and GABARAP are in part degraded with the autophagosome cargo in late endosomes or autolysosomes by lysosomal hydrolases and can reach MHC class II loading. Accordingly, LC3 can be used to deliver antigens to autophagosomes, which frequently fuse with MIICs [9]. This targeting enhances antigen presentation on MHC class II molecules up to 20fold [9, 10]. A physiological condition, under which this self-antigen processing by macroautophagy seems to play a role is thymic selection. Both positive and negative T cell selection was affected in Atg5 negative thymi [11]. During these processes intracellular self-proteins are thought to be presented on MHC class II molecules of thymic epithelial cells (TECs) to ensure low affinity interactions of the selected T cell receptors during positive selection, and eliminating high-affinity T cell receptors against self-proteins during negative selection [12]. Macroautophagy deficiency changes the repertoire of MHC class II presented ligands so that some T cell receptor specificities no longer efficiently survive positive selection, and an autoreactive T cell repertoire emerges from faulty negative selection. Accordingly, autophagosomes fuse frequently with MIICs in TECs [13], and macroautophagy substrates, like mitochondrial proteins and LC3 fusion proteins can induce negative selection [14]. Thus, self-antigens gain access to MHC class II presentation in part via macroautophagy, and this process assists thymic T cell selection.

Following these indications that macroautophagy might contribute to intracellular self-antigen loading onto MHC class II molecules, several viral and bacterial antigens have been investigated for their processing via macroautophagy for CD4⁺ T cell stimulation. Among these, the nuclear antigen 1 of the Epstein Barr virus (EBNA1) was found to be intracellularly processed for MHC class II presentation [15]. This processing involved lysosomal degradation, and upon inhibition of lysosome acidification EBNA1 accumulated in double membrane vesicles

[16]. Moreover, siRNA mediated silencing of Atg12 compromised EBNA1 specific CD4⁺ T cell recognition of EBV infected B cells [16]. EBNA1 processing for MHC class II presentation was enhanced after blocking its access to the nucleus by mutating the nuclear localization domain of EBNA1 [17]. This also broadened macroautophagic antigen processing to additional EBNA1 epitopes. Therefore, physiological levels of EBNA1 are processed for MHC class II presentation by macroautophagy.

Apart from this viral protein, bacterial antigens are processed via this pathway. The transposon-derived neomycin phosphotransferase II (NeoR) gets intracellularly processed by lysosomal hydrolysis for MHC class II presentation [18]. This processing is sensitive to pharmacological macroautophagy inhibitors, and influenza hemagglutinin epitope fusion to NeoR augments MHC class II presentation of this epitope [10, 18]. Interestingly, forced nuclear localization of NeoR does not compromise macroautophagy mediated processing of this antigen for MHC class II presentation [19]. Therefore, nuclear localization might not protect from macroautophagy for all antigens in all cellular backgrounds similarly well. Nevertheless, cytosolic localization probably favors access of antigen to autophagosomes and processing for MHC class II presentation via this pathway. Accordingly, proteins that are injected into the cytosol by bacteria have been described to fall prey to macroautophagy, followed by MHC class II presentation. Along these lines mycobacterial Ag85B is better presented after macroautophagy stimulation by MHC class II molecules of DCs, which then upon adoptive transfer prime more efficiently CD4⁺ T cell responses in vivo [20]. Furthermore, antigens fused to YopE, a Yersinia protein that is injected into the cytosol, are intracellularly processed for MHC class II presentation by a mechanism involving lysosomes and macroautophagy [21]. Thus, bacterial antigens, especially those that escape endosomes, can be processed via macroautophagy for MHC class II presentation.

In addition to this role for macroautophagy in intracellular antigen delivery to MHCs, another autophagic pathway has been implicated in autoantigen delivery for MHC class II

presentation. This pathway is called chaperone-mediated autophagy (CMA) and transports cytosolic proteins for degradation into lysosomes via a translocon in the lysosomal membrane [22]. While this pathway might be avoided by pathogens due to its signal peptide dependency, whose motifs could be easily mutated in pathogen-derived proteins, it has been implicated in antigen processing of autoantigens onto MHC class II molecules. Glutamate decarboxylase 65 (GAD65) and the mutant human immunoglobulin κ light chain SMA, autoantigens in diabetes mellitus and autoimmune hepatitis, respectively, were found to be better intracellularly processed for MHC class II presentation after overexpression of Lamp2a, a lysosomal transmembrane protein that is an integral part of the CMA translocon [23]. Thus, autoantigens that carry a signal peptide for CMA can be intracellularly processed for MHC class II presentation by pathways independent of macroautophagy.

3. Modification of extracellular antigen processing for MHC class II presentation by autophagy

Consistent with this contribution of macroautophagy to antigen processing for MHC class II antigen presentation, diminished herpes simplex virus (HSV) specific CD4⁺ T cell responses and compromised immune control of HSV was observed in mice with Atg5 deficient CD11c⁺ cells, presumably DCs [24]. However, in this study it was also noted that macroautophagy deficient DCs presented different formulations of extracellular antigen less efficiently to CD4⁺ T cells, and that the acquisition of active lysosomal hydrolases by endosomes was delayed in the absence of macroautophagy. These findings were consistent with an earlier report, suggesting that the core machinery of macroautophagy, which executes the Atg8 lipidation, was involved in phagosome maturation [25]. This study suggested that cargo that engages pathogen-associated molecular pattern (PAMP) receptors, like TLR2, recruits the Atg8 ortholog LC3 to the phagosomal membrane that surrounds it after uptake (Figure 1). Furthermore, this LC3 associated phagocytosis (LAP) was suggested to accelerate fusion with lysosomes and degradation of the

endocytosed cargo. Following this initial observation, also uptake of whole cells, so called entosis, was shown to engage LAP [26]. Furthermore, TIM4, a receptor involved in the uptake of apoptotic cells, and Dectin-1, a receptor that recognizes fungal cell wall components, were also shown to stimulate LAP [27, 28]. Dectin-1 mediated antigen uptake was, moreover, shown to enhance MHC class II presentation via LAP [28]. However, all these studies suggested that LAP accelerates phagocytosed cargo degradation, yet MHC class II antigen presentation had previously been shown to benefit from attenuated endosomal degradation [29, 30]. Therefore, future studies will have to define if the LAP vesicles that lead to MHC class II antigen presentation also show accelerated maturation, and, more generally, by which molecular mechanisms LC3 conjugation to the phagosomal membrane modulates the fate of LAP vesicles.

In addition to the regulation of phagocytosis by the molecular core machinery of macroautophagy, lysosomal hydrolase delivery by autophagosomes has been implicated in autoantigen processing for MHC class II presentation (Figure 1). Citrullinated peptides are generated by peptidylarginine deiminases (PADs) and recognized by the adaptive immune system during rheumatoid arthritis [31]. MHC class II presentation of citrullinated epitopes of the model antigen hen egg lysozyme has been described to require macroautophagy [32]. PAD2 and 4, which are expressed in the immune system, seemed to be delivered by autophagosomes to endosomes for the generation of the citrullinated T cell epitopes. Therefore, macroautophagy can also assist extracellular antigen processing by modulating the content of endosomes and lysosomal hydrolysis.

4. Contribution of autophagy to MHC class I antigen presentation

While LAP and intracellular antigen processing by macroautophagy seem to modulate MHC class II presentation for a subset of antigens, the role of macroautophagy for MHC class I presentation is much less understood. Initially, it seemed like antigen targeting to autophagosomes [9] and compromising macroautophagy in DCs or TECs in vivo [11, 24] did not

significantly influence antigen processing for MHC class I presentation. However, recent studies have implicated this pathway in MHC class I antigen presentation under certain circumstances, so far, however, only in vitro. Initially it was reported that lysosomal degradation contributed to MHC class I presentation of HSV glycoprotein B late during HSV infection of human macrophages (>10h) [33]. This late processing was sensitive to pharmacological inhibition and Atg5 silencing, and could be stimulated by macroautophagy induction. These results suggested that during infections that compromise classical antigen processing onto MHC class I molecules by immune evasins, like for example TAP blocking, a vacuolar pathway might take over. This pathway can either transport antigen or MHC class I molecules for vacuolar loading (Figure 2). Similarly, TAP independent antigen processing of the human cytomegalovirus (HCMV) protein pUL138 for MHC class I presentation was found to require macroautophagy [34]. While, however, HSV gB processing was still proteasome dependent, HCMV pUL138 degradation was proteasome independent, suggesting that maybe an endosomal MHC class I loading compartment requires macroautophagy for its formation, and can receive input from lysosomal or proteasomal antigen processing. Supporting this notion is the recent finding that cross-presentation of the F protein of the respiratory syncytial virus (RSV) also requires macroautophagy, but is independent of TAP and proceeds via lysosomal degradation [35]. Indeed, MHC class I molecules can reach endosomal loading also in TAP deficient cells at low temperatures, and then cross-presentation of exogenous antigen becomes TAP independent [36]. In addition to endosomal loading of viral antigens onto MHC class I molecules, a recent study argues in favor of a contribution of macroautophagy to endosomal processing of MHC class I epitopes in the case of chlamydia infection [37]. Indeed in Chlamydia infected DCs, an endosomal route of antigen loading onto MHC class I molecules that is both TAP and proteasome dependent might be supported by autophagosomes. However the contribution of macroautophagy in the processing of bacterial epitopes was only demonstrated by pharmacological inhibitors and by colocalization analysis via confocal microscopy. Therefore,

this observation needs to be further substantiated by more specific macroautophagy inhibition targeting Atgs. Nevertheless, MHC class I molecules might be loaded endosomally under conditions of pathogen mediated blockade of the classical MHC class I antigen processing pathway. While recent studies suggest that macroautophagy supports this endosomal MHC class I loading, it remains so far unclear if transport of MHC class I molecules to an endosomal loading compartment or antigen degradation for MHC class I loading benefits from this pathway.

5. Packaging of antigens via autophagy for antigen presentation

In addition to delivery of cytoplasmic constituents for lysosomal degradation, macroautophagy has also been implicated in signal peptide independent secretion. Exocytosis via macroautophagy was first reported in yeast [38, 39]. In these studies acyl coenzyme A-binding protein (ACBP) was shown to exit cells in a macroautophagy dependent manner, requiring the fusion machinery for the plasma membrane, but not the lysosomal vacuole. Similarly, it has been suggested that IL-1 β , a cytokine that is also secreted by a non-conventional pathway, is released by macroautophagy mediated exocytosis [40]. Finally, amorphous extracellular deposits in age related macular degeneration, so called drusen, also contains autophagosome markers [41]. The releasing retinal pigment epithelium displays increased macroautophagy, decreased lysosomal activity and elevated exocytosis, which could indicate augmented exocytosis via macroautophagy. Such exocytosis could be beneficial for antigen cross-presentation by packaging cytoplasmic material optimally for uptake by bystander antigen presenting cells (Figure 2). Along these lines it has been found that influenza A virus and tumor antigens were only efficiently cross-presented onto MHC class I molecules, when the antigen donor cells were macroautophagy competent [42, 43]. Influenza A virus blocks autophagosome maturation and the accumulating autophagosomes contain viral antigens [44]. This arrest could promote exocytosis. Similarly, autophagosomes from tumor cells lines contain tumor antigens [43], and the inner autophagosomal membrane plus this tumor antigen cargo could be released

for cross-presentation via exocytosis (Figure 2). Therefore, macroautophagy might assist the release of antigens for cross-presentation on MHC class I molecules to T cells.

6. Regulation of lymphocyte function via autophagy

In addition to influencing T cell education by antigen presentation during positive and negative selection in the thymus, macroautophagy also cell-intrinsically influences lymphocyte differentiation and function. Both immature T and B cells seem to require macroautophagy during their development [45-47]. Altered B cell differentiation in the absence of macroautophagy does, however, only lead to decreased B1 cell numbers [47], while overall B cell reconstitution is not significantly changed [45]. Upon further differentiation to plasma cells, however, macroautophagy is required for the maintenance of these antibody production plants [48, 49]. Atg5 deficient plasma cells were found to suffer from expanded ER and increased unfolded protein response stress, leading to their elevated cell death. In immature T cells, so called thymocytes, macroautophagy seems to protect from toxic reactive oxygen species (ROS) production by decreasing mitochondrial mass [50, 51]. Macroautophagy up-regulation for mitochondrial clearance seems to be achieved through activation of the transient receptor potential vanilloid 1 (TRPV1) via NADPH oxidase produced ROS, which activate adenosine monophosphate-activated protein kinase (AMPK) to inhibit mammalian target of rapamycin (mTOR) to activate macroautophagy [52]. In addition, ROS inhibit Atg8 deconjugation by Atg4 to sustain autophagosomal membrane formation [53]. In contrast to these now even mechanistically well understood effects of macroautophagy loss during adaptive lymphocyte development, it remains less clear to which extent this affects the function of these cells in the periphery. While it was originally reported that T cell proliferation after T cell receptor stimulation is compromised in the absence of macroautophagy [46], a more recent study suggested that mature T cells were able to expand efficiently with diminished macroautophagy [45]. Therefore, it seems clear that macroautophagy protects from cell death during T and B cell differentiation, but

its prosurvival role during adaptive lymphocyte activation seems less essential with the exception of plasma cell differentiation.

While physiological macroautophagy levels protect from cell death in most instances, too dramatic induction of this pathway or overexpression of some Atgs can instead compromise cell survival. Along these lines, macroautophagy seemed to contribute to cell death in Th2 polarized CD4⁺ T cells, which display higher autophagosome numbers than Th1 polarized CD4⁺ T cells [54]. Moreover, overexpression of Atg6 sensitizes thymocytes for apoptosis induction [55]. However, this observation could also be explained by the sequestration of anti-apoptotic Bcl-2 by Atg6. Thus, macroautophagy protects from cell death during development, but can diminish or enhance lymphocyte function after maturation. The characteristics of immune responses with these different requirements for macroautophagy need to be determined in the future to predict the outcome of macroautophagy regulating interventions on protective or pathogenic lymphocytes.

7. Conclusions and outlook

Recent studies have revealed that the molecular machinery of macroautophagy does not only serve the purpose of transporting cytoplasmic constituents for lysosomal degradation, but also influences vesicular transport pathways in a broader sense. Indeed, in addition to autophagosome generation, macroautophagy seems to modulate endo- and exocytosis. Moreover, the intimate connections with the cell death machinery, exemplified by the complex formation of Atg6 with Bcl-2, always affects also survival of the macroautophagy regulating cell, in parallel to vesicular transport. Future research will need to characterize how this modular format of the macroautophagy machinery can be specifically recruited to fulfill these different tasks and how one might selectively regulate these different functions of macroautophagy for therapeutic benefit.

Acknowledgments

Research in our laboratory is supported by the National Cancer Institute (R01CA108609), the Sassella Foundation (10/02, 11/02 and 12/02), Cancer Research Switzerland (KFS-02652-08-2010), the Association for International Cancer Research (11-0516), KFSP^{MS} and KFSP^{HLD} of the University of Zurich, the Vontobel Foundation, the Baugarten Foundation, the EMDO Foundation, the Sobek Foundation, Fondation Acteria, Novartis and the Swiss National Science Foundation (310030_143979 and CRSII3_136241).

Conflict of interest statement

The authors declare no conflict of interest with the discussed topics.

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Figure legends**Figure 1. Contributions of macroautophagy to MHC class II antigen processing.**

Macroautophagy can deliver intracellular antigens to MHC class II containing compartments (MIICs) for lysosomal antigen processing and loading of antigen fragments onto MHC class II molecules (top left). In addition, the macroautophagy core machinery modifies phagosomes for LC3-associated phagocytosis (LAP). This can facilitate extracellular antigen processing for MHC class II presentation (top right). Finally, macroautophagy delivers cytosolic hydrolases, like peptidylarginine deiminases (PADs) to endosomal compartments for antigen modification, resulting in MHC class II presentation of altered peptide ligands, like citrullinated peptides (bottom).

Figure 2. Contributions of macroautophagy to MHC class I antigen processing.

Macroautophagy in antigen donor cells facilitates better cross-presentation by presumably supporting antigen exocytosis. After uptake by neighboring antigen presenting cells the transferred antigen is processed and loaded in MHC class I cross-presenting compartments (top left). MHC class I molecules or extracellular antigens might reach these compartments with assistance of the macroautophagic core machinery (top right). In addition, intracellular antigens might be delivered with or without preprocessing via proteasomes to such vesicular MHC class I cross-presenting compartments via classical macroautophagy (bottom).

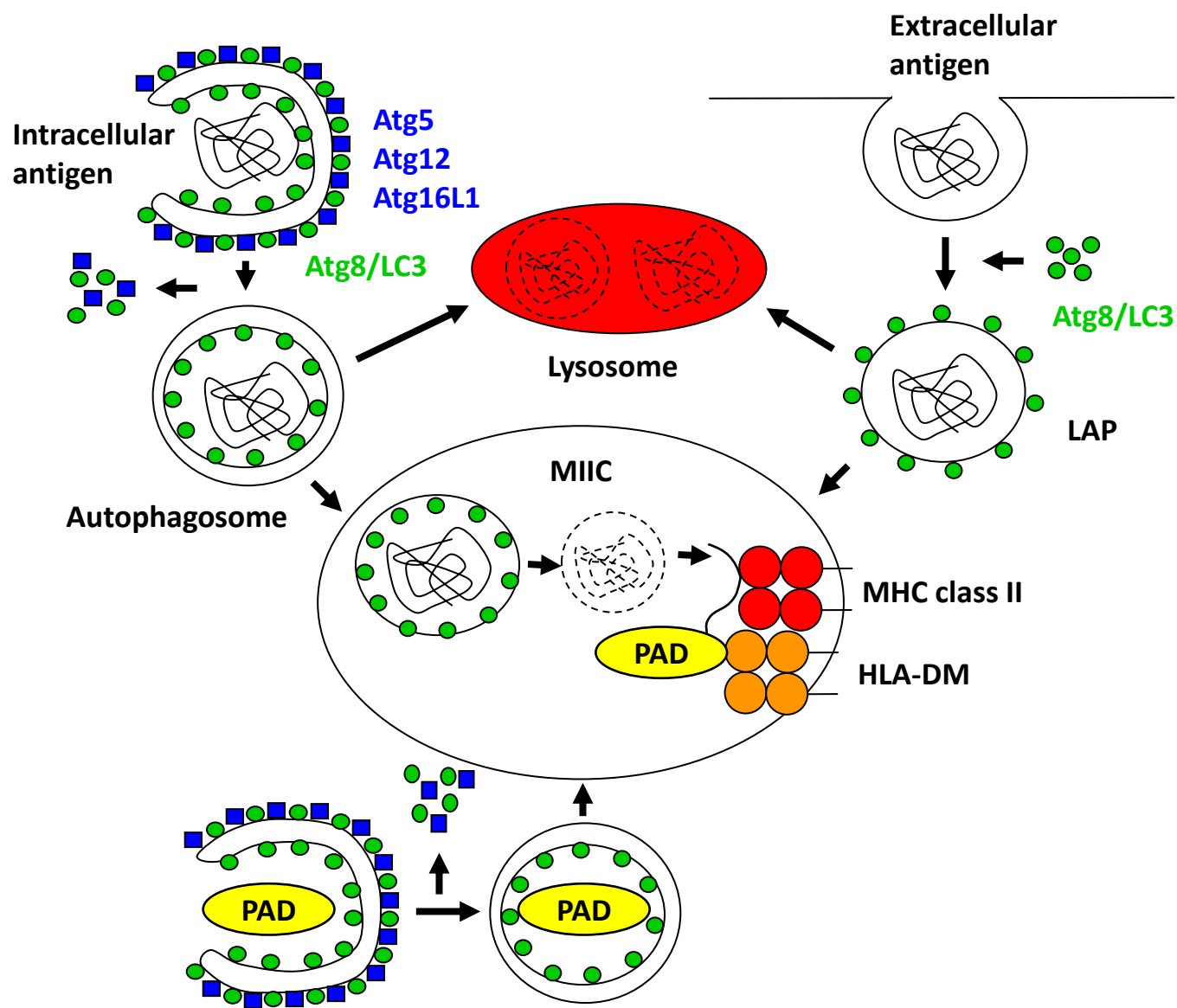


Figure 1

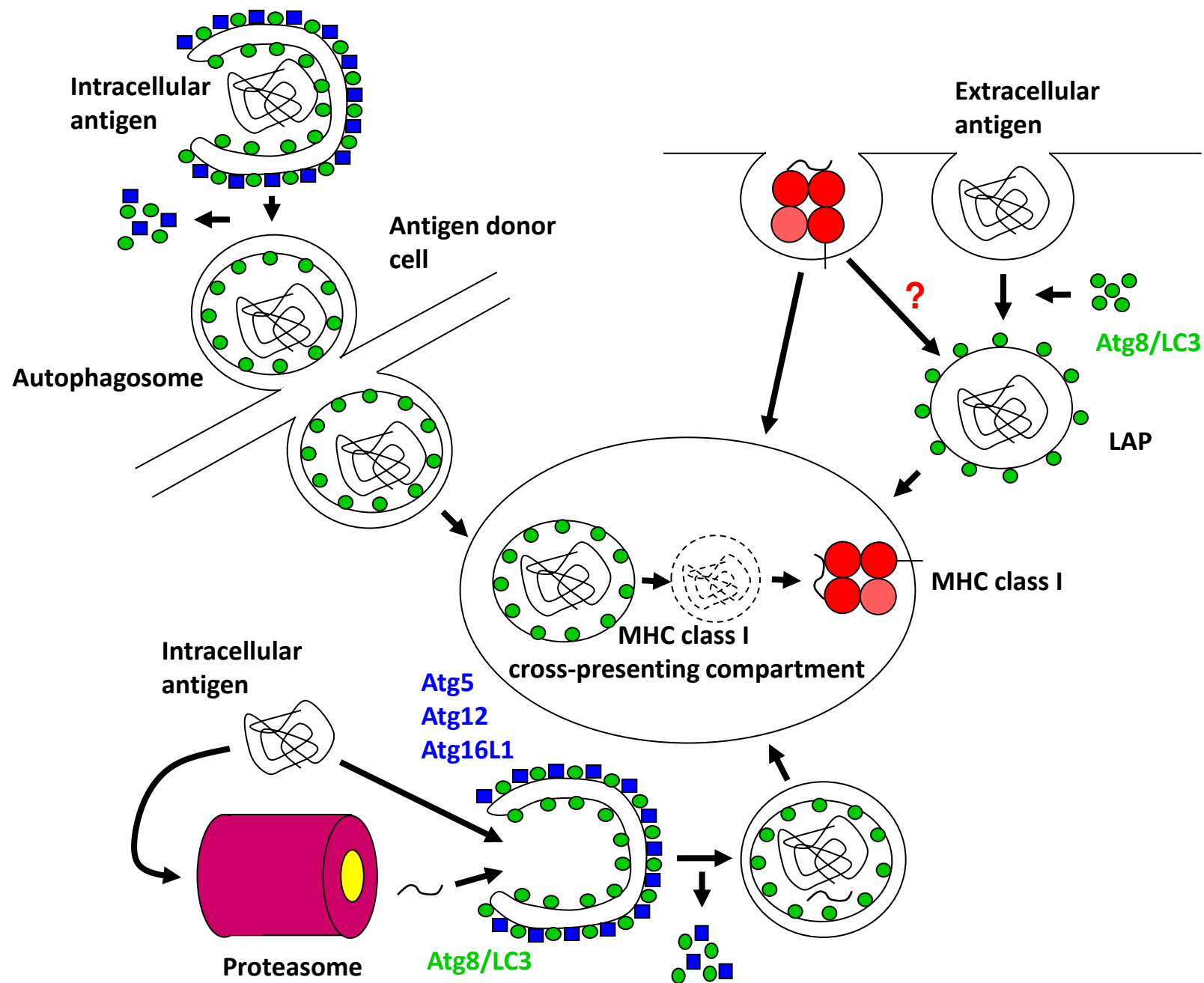


Figure 2